PBPK Modeling/Monte Carlo Simulation of Methylene Chloride Kinetic Changes in Mice in Relation to Age and Acute, Subchronic, and Chronic Inhalation Exposure

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During a 2-year chronic inhalation study on methylene chloride (2000 or 0 ppm; 6 hr/day, 5 days/week), gas-uptake pharmacokinetic studies and tissue partition coefficient determinations were conducted on female B6C3F$_1$ mice after 1 day, 1 month, 1 year, and 2 years of exposure. Using physiologically based pharmacokinetic (PBPK) modeling coupled with Monte Carlo simulation and bootstrap resampling for data analyses, a significant induction in the mixed function oxidase (MFO) rate constant (V_{maxc}) was observed at the 1-day and 1-month exposure points when compared to concurrent control mice, while decreases in glutathione *S*-transferase (GST) rate constant (K_{fc}) were observed in the 1-day and 1-month exposed mice. Within exposure groups, the apparent V_{maxc} maintained significant increases in the 1-month and 2-year control groups. Although the same initial increase exists in the exposed group, the 2-year V_{maxc} is significantly smaller than the 1-month group ($p < 0.001$). Within-group differences in median K_{fc} values show a significant decrease in both 1-month and 2-year groups among control and exposed mice ($p < 0.001$). Although no changes in methylene chloride solubility as a result of prior exposure were observed in blood, muscle, liver, or lung, a marginal decrease in the fat:air partition coefficient was found in the exposed mice at $p = 0.053$. Age related solubility differences were found in muscle:air, liver:air, lung:air, and fat:air partition coefficients at $p < 0.001$, while the solubility of methylene chloride in blood was not affected by age ($p = 0.461$). As a result of this study, we conclude that age and prior exposure to methylene chloride can produce notable changes in disposition and metabolism and may represent important factors in the interpretation of toxicologic data and its application to risk assessment. *Key words*: bootstrap resampling, glutathione *S*-transferase, methylene chloride, Monte Carlo simulation, physiologically based pharmacokinetic modeling, risk assessment. *Environ Health Perspect* 104:858–865 (1996)

In the early 1980s, physiologically based pharmacokinetic (PBPK) modeling was applied to study the disposition of environmental chemicals in mammalian systems (*1*). Through their implicit biological descriptions of mammalian systems, these models provide a functional relationship between external measures of chemical exposure and internal target tissue measures of exposure to the actual toxic moiety (*2*).

One of the earliest chemicals to be studied using PBPK modeling was methylene chloride (1,2-dichloromethane, CH_2Cl_2) (*3*). Based on computer simulation, the *a priori* predictions of blood concentrations were consistent with actual experimental results from three laboratories on four different species, including humans, via two routes of exposure (*3*). Because of the versatility of the PBPK model and its ability to extrapolate among species and routes, Andersen et al. (*3*) expanded the analysis by correlating tumor incidences with target tissue dose based on the putative mechanisms of toxicity from the mixed function oxidase (MFO) and glutathione *S*-transferase (GST) metabolic pathways. Andersen et al. (*3*) concluded that the amount metabolized by the GST pathway correlates best with tumor indices and that conventional risk analysis techniques overestimate human risk when exposed to low concentrations of methylene chloride.

While PBPK modeling is a powerful tool for making *a priori* predictions with limited experimental data (i.e., based on educated assumptions), it is important to challenge the validity of assumptions with proper experimentation. Since pharmacokinetic studies are usually done on young, healthy untreated animals, the potential effects of aging and prior exposure (short-term or chronic) on the kinetic behavior of a chemical are largely unknown and may lead to false conclusions when applied to long-term studies. For example, Yang et al. (*4*) described pharmacokinetic studies on ethylenediamine in Fischer 344 rats that paralleled a 2-year chronic toxicity/carcinogenicity study. These investigators observed an inverse age-related change in volume of distribution that was attributed to the disproportional increase of body fat with age and the water soluble nature of ethylenediamine. Therefore, even though the dose was maintained on the same milligram per kilogram basis (according to body weight) as in conventional toxicology studies, older rats actually had higher levels of exposure to the test chemical due to higher plasma levels of ethylenediamine (*4*). Investigations such as the one reported by Yang et al. (*4*) are useful in the interpretation of toxicologic findings of chemicals of interest, particularly in chronic bioassay studies. Such sentiment is shared by other investigators in the field who stress the need to evaluate chemical toxicity with respect to age and prior exposure (*5–7*).

In 1988, the National Institute of Environmental Health Sciences/National Toxicology Program (NIEHS/NTP) initiated research in which the possible relationships between tumorigenesis, oncogene activation, and cell turn-over rates in methylene chloride-exposed B6C3F$_1$ female mice were investigated (inhalation route; one group only at 2000 ppm) (*8*). Much of the information from this research program has been published in a series of papers in a single issue of *Carcinogenesis* (*8–13*). As an integral part of this investigation, a pharmacokinetic study was incorporated to determine the possible age- and chronic dosing-related kinetic changes. The pharmacokinetic study was designed to occur in parallel with the carcinogenicity studies and enhance the information derived from the study as a whole. Thus, the

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primary objective of this study was to determine changes in metabolic and solubility parameters of methylene chloride as a function of age and chronic exposure.

Materials and Methods

Test Chemical, Animals, and Chronic Inhalation Exposure

Spectroscopic grade methylene chloride (>99% purity) was purchased from Burdick and Jackson Company. B6C3F$_1$ female (C57B1/6N × C3H/HeN MTV-) mice were produced under barrier conditions at Charles River Co. (Portage, MI) under contract to NTP. All animals were 4–6 weeks of age upon arrival and housed individually in H-2000 inhalation chambers (Hazleton Systems, Inc., Aberdeen, MD) 24 hr/day. A complete microbiological/virological health surveillance screening was performed at 6-month intervals and was negative throughout the course of the study. Control and treated animals were given food (NIH-07, Zeigler Brothers, Inc., Gardners, PA) and filtered (0.2 µm), UV-irradiated water *ad libitum*.

Methylene chloride was vaporized in nitrogen, diluted with air, and introduced into whole-body chambers where concentrations were monitored with a Miran 80 infrared spectrometer. Mice were exposed to 2000 ppm methylene chloride during the day at a regimen of 6 hr/day, 5 days/week in the chronic toxicology study for 2 years; control mice were sham-exposed to conditioned HEPA-filtered air. All other information concerning animal care and exposure is presented in detail elsewhere (*14*).

Age- and Chronic Dosing-related Pharmacokinetic Studies

In the pharmacokinetic study, gas-uptake experiments (*15*) were conducted on control and exposed (2000 ppm methylene chloride) female mice at 1 day and 1, 12, and 24 months following initiation of exposure regimen. At each of the designated time points, 24 control or exposed mice from the 1-day or 1-month group or 12 control or exposed mice from the 12- or 24-month group were placed in a 12-liter closed recirculating exposure system. The differences in numbers among different age groups reflect our attempt to provide equivalent liver mass in the gas-uptake system for methylene chloride disposition. Oxygen was added and maintained at approximately 21% while carbon dioxide was removed with a barium hydroxide scrubber. Methylene chloride was added in sufficient quantity to achieve a targeted initial chamber concentration and the atmosphere was serially sampled by an in-line Hewlett Packard 5890 gas chromatograph (Hewlett Packard, Avondale, PA) for up to 6 hr.

Each group of mice (control or exposed at various time points) was exposed to an initial methylene chloride concentration of 500 ppm in the gas-uptake system. After 48 hr, the same batch of animals was again exposed in the gas-uptake system at an initial concentration of 1000 or 2000 ppm methylene chloride. Although the primary reason for such a repeated exposure design was the limited availability of animals within a complex study design (*14*), we felt that the initial gas-uptake studies at 500 ppm would not have a major impact on the overall pharmacokinetic experimental outcome for the following reasons: concurrent control animals were treated identically to exposed animals; the methylene chloride-treated group was exposed to 2000 ppm for 6 hr/day, 5 days/week, and pharmacokinetic experiments were conducted in the latter part of the week to allow at least 2 exposure days prior to pharmacokinetic experiments; and 500 ppm is the initial concentration at time-zero of the gas-uptake experiment, and the concentration in the chamber decreases rapidly due to disposition and biotransformation. Thus, the animals were exposed to lower and lower concentrations of methylene chloride, which were not believed to cause significant physiological changes as compared to the daily exposures at 2000 ppm. Further, whatever changes would occur would presumably be present in the concurrent control mice as well. Finally, due to the short half-life of methylene chloride in the body, it was believed that a 48-hr resting period would be sufficient for the clearance of residual methylene chloride in the mouse.

Following the final gas-uptake study, mice were sacrificed and organ masses such as liver and lung mass were measured. The organs and remaining tissues such as muscle and fat were dissected and homogenized for tissue partition coefficient measurements to examine potential physiological changes related to disposition. In all studies conducted in this project, the order of experimentation was from low to high concentrations of methylene chloride and from control to exposed mice.

Determination of Tissue Partition Coefficients

A modified version of the vial equilibration technique of Sato and Nakajima (*16*) was used to determine tissue partition coefficients for methylene chloride in mice at various exposure periods on this project (*17,18*). Head space analyses of methylene chloride were made using a Hewlett Packard 5790A GC (Hewlett Packard) with a flame ionization detector. The column was 10% SE-30 on Supelcoport 80/100 mesh (10 ft, 1/8 in O.D., S.S.) (Supelco Inc., Bellefonte, PA) and resulted in an average retention time of 1.1 min for methylene chloride. Oven temperature was isothermal at 80°C while detector and injector temperatures were maintained at 300° and 125°C, respectively. Flow rates were as follows: air (390 ml/min), hydrogen (18 ml/min), and nitrogen (32 ml/min).

Physiological Pharmacokinetic Model

The basic methylene chloride model structure was described by Andersen et al. (*3*) (Fig. 1). The body was subdivided into five tissue groups consisting of the liver, lung, slowly perfused, rapidly perfused, and fat. Each compartment was represented by mass balance differential equations which incorporate blood flows, partition coefficients, and tissue volumes. The concentration of methylene chloride in blood leaving the lung was assumed to be in equilibrium with the concentration in alveolar air as determined by the blood:air partition coefficient. Methylene chloride was distributed to all tissues and eliminated by metabolism in the lung and liver, as well as by exhalation.

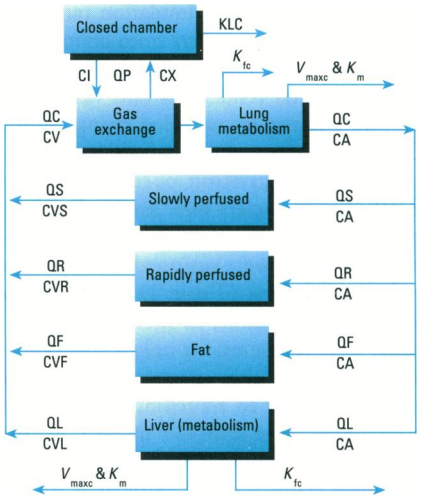

Figure 1. Diagram of the physiologically based pharmacokinetic model used for methylene chloride. Tissues of the body are grouped into five compartments with flow and partition coefficients: lung, fat, liver, richly perfused, and slowly perfused. Methylene chloride enters the body through inhalation (CI) with absorption into pulmonary blood in the gas-exchange compartment. Concentrations of methylene chloride in the venous blood (CV) and arterial blood (CA) are calculated based on blood flow (QC, QS, QR, QF, and QL), alveolar ventilation (QP), and solubility information. Exhalation (CX) and metabolic processes, such as Michaelis-Menton metabolism (V_{maxc} and K_m) or first-order enzymatic conjugation (K_{fc}), are included for removal of methylene chloride. Loss from the chamber (KLC) is also accounted for.

The basic model structure was modified for a combination of Monte Carlo simulation and bootstrap resampling using a method described by Thomas et al. (*19*). Briefly, a separate program was written to randomly sample model parameters from defined distributions or, in the case of bootstrapped parameters, resample parameters with replacement from given data sets. A total of 1000 values were sampled for each parameter and stored in a format recognized by the differential equation solver (SimuSolv Software, Dow Chemical Co., Midland, MI). The model was then run for each set of parameters while the metabolic constants, V_{maxc} (mixed function oxidase rate constant) and K_{fc} (GST rate constant), were optimized based on the gas-uptake data using a least-squares technique. Following optimization, data values were stored for statistical analysis. For model stability as well as practical reasons, the sum of the individual organ masses was constrained to be less than the body mass by setting the volume of slowly perfused tissue equal to the difference between body mass and the remaining tissues.

The Monte Carlo method is a form of uncertainty analysis that allows the propagation of uncertainty through a model which results in an estimate of the variance in model output (*19*). For the uncertainty analysis, Monte Carlo simulation randomly samples model parameters from defined distributions (e.g., normal, lognormal) with defined central tendencies and variability. Among parameters sampled using Monte Carlo simulation, the distributions of cardiac output, partition coefficients, and metabolic parameters were assumed to have lognormal distributions while the remaining parameters were assumed to have normal distributions (Table 1) (*20*). Tissue partition coefficients, as mentioned previously, were measured for only a subpopulation of the exposure group; therefore, the parameters used to represent the partition coefficients were sampled using a Monte Carlo routine based on the exposure-specific central tendency and associated uncertainty for each group. Parameters derived from the literature, such as blood flows and remaining tissue volumes, were also sampled using Monte Carlo simulation and assumed to have a standard deviation of 20% with two exceptions: A1 and A2 (*20*). Parameters A1 and A2 represent the V_{maxc} and K_{fc} ratios between the lung and the liver and were assigned standard deviations of 17 and 12%, respectively (*17*) (Table 1).

In contrast, bootstrap resampling continually resamples model parameters from given data sets and does not assume a given distribution. This allows parameters such as body mass, liver mass, and lung mass, which were measured for all individual mice, to be constrained to represent only those mice producing the gas-uptake data.

Due to the lipophilicity of methylene chloride, the percent of body weight as fat is an important parameter in the modeling process. Consequently, the change of the fat to body weight ratio as a function of age is also important. An estimate of these values was based on experimentally determined data from C57 mice (L. Birnbaum, personal communication) and Diliberto et al. (*21*). For example, C57 mice at 3 or 18 months of age maintained fat to body weight ratios of 7 to 14%, respectively. In our study, the following mean fat to body weight ratios were used for female $B6C3F_1$ mice at various ages: 4% for 8 weeks (1-day group), 10% for 12 weeks (1-month group), 15% for 60 weeks (1-year group), and 12% for 107 weeks (2-year group). The lower value for the 2-year animals was chosen due to their generally lower body weight relative to the 1-year animals.

Age-related changes in alveolar ventilation and cardiac output were also estimated based on previous studies in both rats and mice. According to Pesce et al. (*22*), carbon dioxide emissions (VCO_2) from female OF_1 mice dropped 23% between the ages of 30 and 95 days and 57% between 30 and 412 days. Assuming no change in the partial pressure of alveolar carbon dioxide, the change in carbon dioxide emissions is directly proportional to the change in alveolar ventilation (*23*). In our model, an initial scaled alveolar ventilation of 23 l/hr kg was used for the 1-day group (*24*). Accordingly, the 1-month, 1-year, and 2-year groups were assigned lower ventilation rates of 21, 15, and 15 l/hr kg, respectively.

In contrast to alveolar ventilation for which rates have been measured with age, no previous studies on age-related changes in cardiac output for mice were found. This is due primarily to the invasive nature of the procedure and the relatively small size of mice (*25*). However, using age-related changes in cardiac output in rats as a model, Isoyama et al. (*26*) measured cardiac output of 10.2, 9.8, and 7.9 l/hr kg in 9, 18, and 22-month-old control rats. Although the drop between the 9 and 18-month-old rats was not statistically significant, the decrease in the 22-month-old rats was significant at $p < 0.05$ (*26*). The data by Isoyama et al. (*26*) suggest an initial plateau of cardiac output with age, followed by a decline in the latter ages. Accordingly, cardiac output was set in our model to 16, 16, 15, and 12 l/hr kg for the 1-day, 1-month, 1-year, and 2-year groups, respectively.

Data Treatment

Although techniques commonly applied to toxicologic and pharmacokinetic studies involve the application of statistics for continuous and normally distributed populations, a considerable amount of the optimized metabolic data was such that the underlying distribution was not easily speci-

Table 1. Model parameters used in the PBPK/Monte Carlo simulations for $B6C3F_1$ mice

PBPK parameter	Data type	Mean	SD	Sample distributions
Weights:				
Body (kg)	X	[a]	[a]	—
Lung	X	[a]	[a]	—
Liver	X	[a]	[a]	—
Rapidly perfused	L	0.05[b]	20%	Normal
Slowly perfused	C	—	—	—
Fat	L	[c]	20%	Normal
Flows				
Alveolar (l/hr kg)	L	21	20%	Lognormal
Cardiac output (l/hr kg)	L	—	—	—
Liver	L	0.24[d]	20%	Normal
Rapidly perfused	C	—	—	—
Slowly perfused	L	0.24[d]	20%	Normal
Fat	L	0.05[d]	20%	Normal
Partition coefficients				
Tissue/air	X	[e]	[e]	Lognormal
Metabolic constants				
K_m (mg/l)	L	0.4	20%	Lognormal
A1	L	0.416	0.07	Lognormal
A2	L	0.137	0.016	Lognormal

[a]Experimental data bootstrapped to represent only those mice generating gas-uptake data.
[b]Mean given as a percentage of body weight.
[c]Mean varied with age group.
[d]Mean given as a percentage of cardiac output.
[e]Mean and standard deviation varied with study group.
X, experimental observation; C, calculated; L, literature.

fied and, more importantly, varied from study group to study group. Therefore, results of the optimized metabolic parameters were summarized using the median and interquartile ranges that are relatively stable to the influences of the outliers. Statistical analysis of the optimized metabolic parameters were also performed using nonparametric statistics due to the non-normality of data and differences in the distributions from study group to study group. Changes in the V_{maxc} and K_{fc} among different age groups were assessed with the Kruskal-Wallis nonparametric alternative to a one-way analysis of variance. Between-group post-tests and exposed/control comparisons were performed using the Mann-Whitney-Wilcoxon U test and adjusted using the Bonferroni adjustment for multiple comparisons (*27*). Statistical tests were performed using the Minitab statistical software package, release 7.2 (Minitab, Inc., State College, PA).

Analysis of the partition coefficients with respect to age and treatment was performed using both a parametric two-way analysis of variance and a nonparametric ranked two-way analysis of variance. Both analyses gave, essentially, the same results. Between-group post-tests were performed using the Bonferroni adjustment for multiple comparisons (*27*).

Table 2. Summary of body and organ weights on B6C3F$_1$ mice used in gas-uptake pharmacokinetic studies[a]

Study group		No. of animals	Body weight (g)[b]	Organ weight (g)[c]	
				Liver	Lung
1 day	Control	24	18.3 ± 1.0	0.87 ± 0.18	0.147 ± 0.019
	Exposed	24	18.0 ± 1.0	0.88 ± 0.08	0.139 ± 0.016
1 month	Control	24	22.1 ± 0.8	1.16 ± 0.07	0.172 ± 0.020
	Exposed	24	22.9 ± 1.1	1.25 ± 0.13	0.181 ± 0.022
1 year	Control	12	37.8 ± 4.9	1.42 ± 0.16	0.240 ± 0.030
	Exposed	12	38.0 ± 5.6	1.55 ± 0.17	0.253 ± 0.050
2 year	Control	12	37.8 ± 4.7	2.00 ± 0.41	0.311 ± 0.055
	Exposed	12	36.1 ± 5.4	2.59 ± 0.82	0.344 ± 0.054

[a]Mean ± SD.
[b]These values represent mean body weights at the beginning of the first gas-uptake study in the series.
[c]The organ weights at and beyond 12 months include tumor masses.

Table 3. Summary of values for *in vivo* apparent V_{maxc} (mg/hr kg) following optimization and Monte Carlo simulation for B6C3F$_1$ mice exposed to methylene chloride

Study group		Mean	Median	Standard deviation	First/third quartiles	Skewness	Kurtosis
1 day	Control	4.55	4.53**	0.39	4.29, 4.79	0.42	0.24
	Exposed	6.46	6.44*#	0.63	6.04, 6.84	0.32	0.70
1 month	Control	7.41	7.28**	1.08	6.59, 8.11	0.46	-0.27
	Exposed	9.43	9.31*#	1.36	8.51, 10.37	0.19	-0.31
1 year	Control[a]	[b]	11.04**	[b]	10.17, 12.05	23.9	640.6
	Exposed[a]	[b]	9.76*#	[b]	8.56, 10.73	6.82	49.6
2 year	Control	9.16	9.31**	1.38	8.18, 10.17	-0.20	-0.30
	Exposed	8.57	8.68*#	1.58	7.34, 9.57	0.36	0.47

[a]Results should be interpreted with caution due to inability of model to describe gas-uptake data.
[b]Values not reported due to skewness of results and influence of outliers.
*Statistically significant for control vs. exposed; $p < 0.001$ (as determined by study group).
**Statistically significant between age groups among controls; $p < 0.05$.
#Statistically significant between age groups among exposed; $p < 0.05$.

Table 4. Summary of values for *in vivo* apparent K_{fc} (kg/hr) following optimization and Monte Carlo simulation for B6C3F$_1$ mice exposed to methylene chloride

Study group		Mean	Median	Standard deviation	First/third quartiles	Skewness	Kurtosis
1 day	Control	1.94	1.89**	0.30	1.73, 2.08	1.07	1.41
	Exposed	0.99	0.93*#	0.32	0.78, 1.16	0.73	0.84
1 month	Control	1.13	1.18**	0.41	0.89, 1.41	-0.46	0.13
	Exposed	0.83	0.89*#	0.52	0.41, 1.17	-0.06	-0.73
1 year	Control[a]	[b]	2.67**	[b]	1.13, 3.15	3.58	33.6
	Exposed[a]	[b]	3.01*#	[b]	2.37, 5.14	6.85	49.9
2 year	Control	0.11	0.03**	0.16	0.01, 0.18	1.85	3.91
	Exposed	0.32	0.32*#	0.21	0.16, 0.47	0.20	-0.64

[a]Results should be interpreted with caution due to inability of model to describe gas-uptake data.
[b]Values not reported due to skewness of results and influence of outliers.
*Statistically significant for control vs. exposed; $p < 0.001$ (as determined by study group).
**Statistically significant between age groups among controls; $p < 0.05$.
#Statistically significant between age groups among exposed; $p < 0.05$.

Results

Body Weights, Liver Weights, and Tumors Observed

The data on body and organ (liver and lung) weights for the animals within the pharmacokinetic studies are summarized in Table 2. The mean body weight in the 1-day mice was approximately 18 grams; it peaked during the 12-month exposure point at 38 grams before decreasing to 36 grams in the exposed mice at 24 months. The organ weights at and beyond 12 months included the tumor masses.

Age- and Chronic Dosing-related Pharmacokinetic Changes

Summary statistics for the optimized metabolic constants are presented in Tables 3 and 4 and shown graphically in Figures 2 and 3. The model descriptions of the gas-uptake data are illustrated in Figures 4 and 5. A nonparametric analysis of variance revealed significant differences ($p < 0.05$) among time points for the optimized metabolic rate constants (V_{maxc} for mixed function oxidase pathway and K_{fc} for the glutathione *S*-transferase pathway), as well as significant differences between exposed and control groups ($p < 0.05$). The apparent V_{maxc} and K_{fc} values for the 1-day, 1-month, 1-year, and 2-year exposed groups are all significantly different from those for the concurrent control groups according to between-group post-tests ($p < 0.001$); however, the 1-year metabolic data must be interpreted with caution. The physiological model was unable to adequately describe

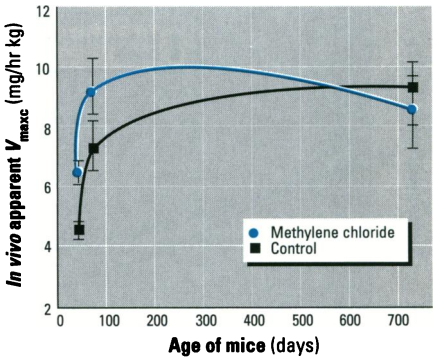

Figure 2. Age-related changes in the apparent maximum metabolic rate for MFO pathway (V_{maxc}) for B6C3F$_1$ mice exposed to 2000 ppm methylene chloride and control mice. Data points represent the median ± first and third quartiles for exposure periods of 1 day, 1 month, and 2 years.

the gas-uptake data, and all modeling attempts to describe the gas-uptake data, at the 1-year time point were poor within all reasonable variations of physiological parameters. Experimentally, personnel, as well as methods and materials, remained constant throughout the 2-year period; therefore, we do not believe this anomally was due to variability in techniques. Because there were examples in which replicate chronic toxicity studies yielded conflicting results, we might be dealing with a larger issue of the lack of homogeneity in the experimental population, despite the use of an inbred strain and proper randomization processes.

Age-related differences in the median values for the control groups are also apparent (Figs. 2 and 3). The apparent V_{maxc} is significantly greater in the 1-month control group when compared with the 1-day control group ($p < 0.001$), and the 2-year control group is significantly larger than the 1-month control group ($p < 0.001$). The same initial increase exists in the exposed groups, but the 2-year exposed V_{maxc} is significantly smaller than the 1-month group ($p < 0.001$). In contrast, age-related differences in the median K_{fc} show a decrease in both the 1-month and 2-year groups ($p < 0.001$) (Fig. 3).

Tissue Partition Coefficients in Relation to Age and Exposure

Tissue partition coefficients determined at various time points during the course of the 2-year study are summarized in Table 5. According to a two-way analysis of variance, significant differences with respect to treatment were not found in the blood:air, muscle:air, liver:air, or lung:air partition coefficients at $p = 0.416$, 0.087, 0.421, and 0.275, respectively. In contrast, previous exposure to methylene chloride had a marginal effect in lowering the fat:air partition coefficient at $p = 0.053$, based on the analysis of variance. Age-related differences were found in muscle:air, liver:air, lung:air, and fat:air partition coefficients at $p < 0.001$ while the solubility of methylene chloride in blood did not change ($p = 0.461$). Between-group Bonferroni post-tests reveal significant differences with respect to age. Specifically, lung:air and fat:air partition coefficients are significantly different between the 1-day and 1-year groups ($p < 0.05$), as well as the 1-month and 1-year groups ($p < 0.05$). All tissues, with the exception of blood, are statistically different in the 2-year group as compared to the 1-day, 1-month, or 1-year groups ($p < 0.05$). It should be noted that tissue partition coefficients for the 1-year time period appear to conflict with the trends apparent in the surrounding time periods. Similar difficulties in describing the gas-uptake data at the 1-year exposure period were also observed and outlined in the previous section. Thus, the 1-year partition coefficient data should also be interpreted with caution.

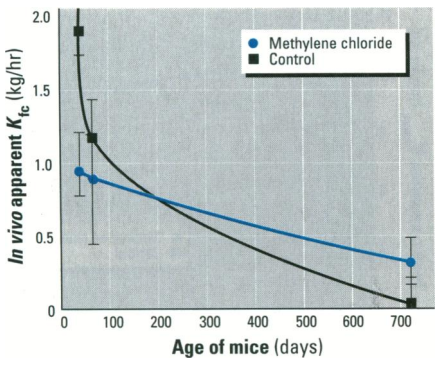

Figure 3. Age-related changes in apparent first-order GST metabolism (K_{fc}) for B6C3F$_1$ mice exposed to 2000 ppm methylene chloride and control mice. Data points represent the median ± first and third quartiles for exposure periods of 1 day, 1 month, and 2 years.

Discussion

Computer Optimized V_{maxc} and K_{fc} Values and Their Significance

While previous PBPK studies have used computer optimization routines for comparing enzyme metabolic parameters *in vivo*, the point estimates obtained by deterministic techniques may not be representative of the actual values within a population and may allow only limited statistical comparisons between dose groups or controls (*17,28,29*). Our study used Monte Carlo and bootstrapping techniques in conjunction with computer optimization to reveal the range of possible values for these metabolic parameters, given the intraspecies variability within the study group. A word of caution must be emphasized in avoiding overindulgence in the utility of the present approach of PBPK modeling/Monte Carlo simulation. Statistical significance is directly related to the size of the sample and, in our study, the number of simulations. Therefore, by increasing the number of simulations, it is possible to increase the statistical significance of two comparison groups beyond that of practical significance. For example, the difference between the median 2-year exposed (8.68) and median 2-year control (9.31) groups for V_{maxc} was found to be statistically significant ($p < 0.001$). While the difference is not necessarily artifactual, a difference of 0.63 would not maintain a practical significance in conventional toxicology studies. Nonetheless, with the possible exception of the 1-year comparisons for exposed and control groups, the remaining values in our study were different from both practical and statistical standpoints.

Significant increases in V_{maxc} were seen at the 1-day and 1-month exposure points when compared to controls and may represent an initial induction of the MFO path-

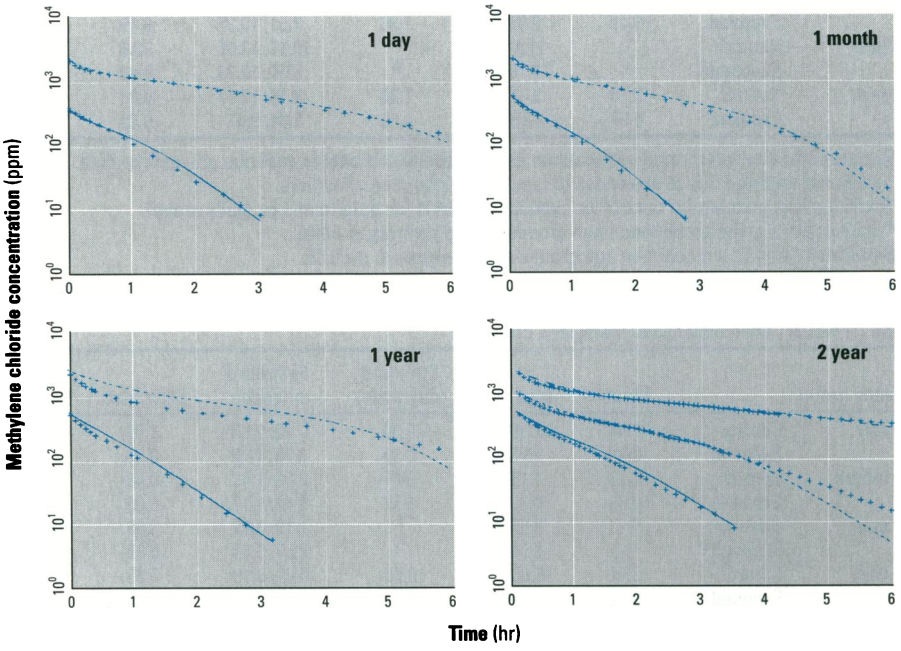

Figure 4. Final gas-uptake pharmacokinetic curves of control B6C3F$_1$ mice used to estimate the kinetic constants for the MFO and GST pathways. Data show the concentration of methylene chloride (ppm) in the chamber atmosphere as a function of time. Experimental data are shown as symbols while the computer model simulation is presented as a solid line.

way (Table 3 and Fig. 2). The extent of the V_{maxc} increase in the 1-day treated animals (6.44) when compared to controls (4.53) reveals a relatively rapid time course of induction for methylene chloride. As a comparison, phenobarbital and 3-methylcholanthrene are known to produce maximal induction at approximately 4 days and 48 hours, respectively (*30*). The difference in V_{maxc} was maintained in the 1-month time period and reversed in the 2-year exposure group; this suggests that the initial induction of MFO activity was maintained as long as 1 month following the beginning of exposure. Because the mice were 4 to 6 weeks of age on arrival, the relatively large difference in control and exposed mice at the 1-day and 1-month exposure points is attributed to the high rate of *de novo* protein synthesis found in relatively young mice. At the 2-year exposure periods, *de novo* protein synthesis is significantly reduced and the toxic effects of continuous exposure to methylene chloride may be exerting itself.

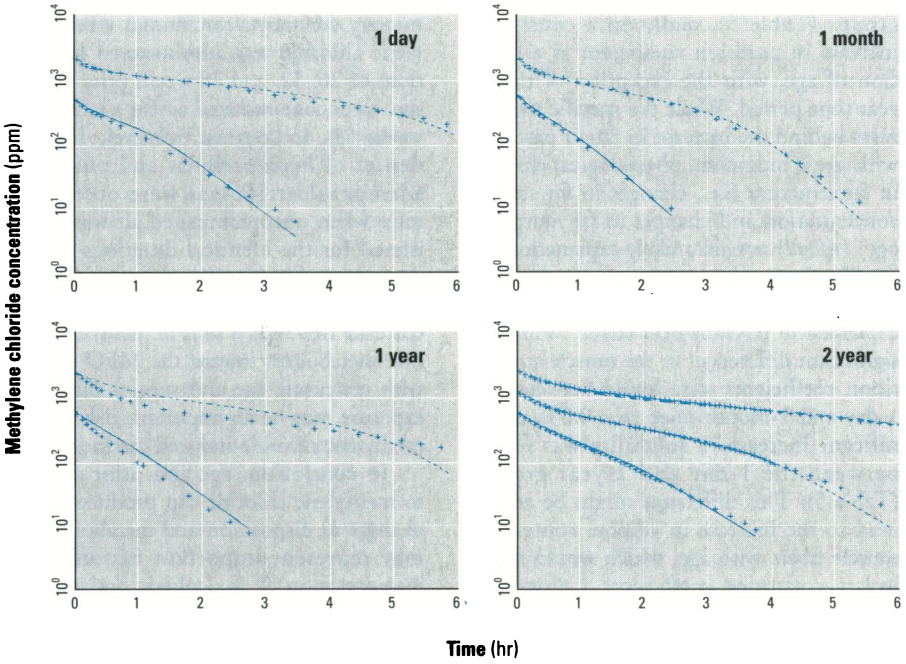

Figure 5. Final gas-uptake pharmacokinetic curves of exposed B6C3F$_1$ mice used to estimate the kinetic constants for the MFO and GST pathways. Data show the concentration of methylene chloride (ppm) in the chamber atmosphere as a function of time. Experimental data are shown as symbols while the computer model simulation is presented as a solid line.

Table 5. Tissue partition coefficients of methylene chloride from female B6C3F$_1$ mice in relation to age and exposure[a,b]

Study group		Blood	Fat[c]	Muscle	Lung	Liver
1 day	Control	26.0 ± 2.1	110 ± 11	14.1 ± 1.6	17.0 ± 1.8	18.6 ± 2.2
	Exposed	27.2 ± 3.2	106 ± 4	14.4 ± 2.8	17.7 ± 3.5	19.5 ± 0.4
	Average[d]	26.6	108*	14.2*	17.4*	19.0*
1 month	Control	26.4 ± 3.3	115 ± 10	13.7 ± 2.4	16.5 ± 4.0	17.4 ± 2.6
	Exposed	25.0 ± 1.3	108 ± 6	13.5 ± 2.1	16.8 ± 3.1	18.2 ± 2.1
	Average	25.7	112*	13.6*	16.6*	17.8*
1 year	Control	24.7 ± 0.7	103 ± 4	13.6 ± 1.8	20.2 ± 1.0	17.9 ± 2.5
	Exposed	25.1 ± 1.5	102 ± 2	14.1 ± 1.8	23.1 ± 3.4	18.0 ± 1.6
	Average	24.9	102*	13.8*	21.6**	18.0*
2 year	Control	25.6 ± 0.6	124 ± 4	22.9 ± 2.6	11.6 ± 1.3	13.6 ± 1.2
	Exposed	27.7 ± 0.4	117 ± 3	27.2 ± 1.7	12.1 ± 0.9	13.9 ± 0.9
	Average	26.6	120**	25.0**	11.8#	13.8**

[a]Mean ± SD.
[b]According to a two-way analysis of variance, age related diferences were found in muscle, liver, lung, and fat at $p < 0.001$.
[c]According to a two-way analysis of variance, previous exposure to methylene chloride had a significant effect on its solubility in fat at $p = 0.053$.
[d]Average of control and exposed groups included to observe trend with age.
*,**,#Significant ($p < 0.05$) differences between age groups as determined by Bonferroni between-group post-tests: all groups with a column with the same symbol are not significantly different, but groups with different symbols are significantly different.

Age-related changes in V_{maxc} within control and exposed groups were also observed. In control animals, V_{maxc} increased approximately 61% between the 1-day and 1-month groups and 106% between the 1-day and 2-year groups. A similar increase was observed in exposed groups between the 1-day and 1-month mice, followed by a decrease in the 2-year animals. As previously reported, methylene chloride metabolism via the MFO pathway occurs predominantly through the P4502E1 isozyme (*31*), and similar age-related changes in P4502E1 activity have been reported elsewhere (*32–34*). Using 7-ethoxycoumarin as a substrate for cytochrome P4502E1 (*35*), Stohs et al. (*32*) reported a near 3-fold increase in liver 2E1 activity for female Swiss Webster mice between the ages of 1 month and 9 months; this increase was followed by a gradual decline in activity out to 18 months. Lung P4502E1 activity showed a similar age-related change with a peak at 6 months (*32*); however, in a limited study of only 12-month and 24-month-old female C3B10RF$_1$ mice, Koizumi et al. (*33*) found no significant change in P4502E1 activity with age.

Although differences in the GST-dependent pathway were also observed between control and treated mice, the gas-uptake system is not extremely sensitive to changes in K_{fc} (*2*), and drawing conclusions as to the meaning of these differences are tenuous. Nevertheless, there appear to be consistent decreases in apparent K_{fc} within exposure groups (Fig. 3) and in the apparent K_{fc} of the exposed mice in the 1-day and 1-month groups relative to the controls (Table 4). In the literature, support for a change in GST metabolism with age is inconsistent and depends on the substrate, sex, and strain involved. Studies by Carrillo et al. (*36*) in female C57 black mice reveal a decline in basal GST activity using 1,2-dichloro-4-nitrobenzene (DCNB) as a substrate, while activity toward 1-chloro-2,4-dinitrobenzene (CDNB) remains relatively constant. Substrate specificity for DCNB is related to GST MIII (class mu) (*37*), which suggests a decline in this isozyme with age. Other studies by Egaas et al. (*38*) reveal an age-dependent increase in GST MI (class alpha) activity between late adolescent C57BL/6 mice (44 days old) and sexually mature mice (88 days old) while female DBA/2 mice showed a decline in GST MII (class pi) activity. For methylene chloride metabolism, the GST class theta has been identified as the primary isozyme in humans and rats (*39*); however, no information in the literature was found on the

change in GST class theta activity with age.

The changes in apparent K_{fc} due to prior exposure are also without precedence in the literature. Green (*6*) reported extensive vacuolation of the smooth endoplasmic reticulum (SER) of the Clara cells in the lungs of male B6C3F$_1$ mice following a single inhalation exposure of methylene chloride at 4000 ppm; however, after 10 days of continuous exposure, the vacuolation was no longer apparent. Concurrent enzyme profile studies revealed the remarkable effect that cytochrome P450 metabolism of methylene chloride decreased by about 50% in lung microsomes in both the 1-day and 10-day exposed mice, whereas GST metabolism remained unchanged in the lung cytosolic fraction (*6*). In our study, the exposure level was 2000 ppm methylene chloride, and whether similar effects on Clara cells in the lung existed remains unknown. Since lung metabolism of methylene chloride represents only a small fraction of the overall metabolism, any time-course and exposure-related changes that may have occurred would have been totally masked by the predominance of liver metabolism in our *in vivo* pharmacokinetic studies. Nevertheless, in considering the carcinogenic effects of methylene chloride, such organ specific time-course and exposure-related pharmacokinetic changes are of utmost importance.

Changes in Tissue Partition Coefficients with Age and Exposure

While prior exposure to methylene chloride produced only a marginal difference in the fat:air partition coefficient at $p = 0.053$, the effect of treatment resulted in a consistent reduction (~5%) in the fat:air partition coefficient in all exposure time points, with the exception of the 1-year group (Table 5). However, this reduction may be somewhat artificial and might be attributed to the headspace analysis used to determine partitioning. When partition coefficients are measured *in vitro*, a known amount of methylene chloride is injected into a closed vial containing a known amount of tissue. Following equilibration, the headspace is then analyzed for methylene chloride while the amount in the tissue is inferred from subtraction. Therefore, if animals were exposed to methylene chloride prior to sacrifice and a significant quantity of methylene chloride remained in the fat, the partition coefficient may be artificially reduced because the total quantity of methylene chloride in the vial and tissue is greater than the known amount added to determine partitioning. Fortunately, this phenomenon produced only a marginal effect in the fat tissue and had no significant effect in the other tissues studied. In retrospect, one possible solution to this problem may be to measure methylene chloride concentrations in both the tissue and the headspace.

Age-related differences in solubility were observed in the muscle, liver, lung, and fat tissues, while the blood:air partition coefficients maintained no significant change with age. These results are consistent with the physiological changes that occur in the majority of tissues during the aging process. The fat:air partition coefficients, when averaged across exposure groups (Table 5), maintain a consistent increase in partition coefficient as a function of age, with the exception of the 1-year time period. While the specific mechanism behind the increase in fat:air partition with age is unknown, physiological changes in fat structure (i.e., changes in fat steroid concentration and changes in fat morphology) (*40,41*) remain a likely explanation.

Changes in the average muscle:air partition coefficient with age can also be explained in physiological terms. While no significant differences in the muscle:air partition coefficients were found between the 1-day and 1-month mice ($p < 0.05$), a significant increase in solubility was found between the 1-day and 2-year groups (Table 5). This difference might be attributed to the increase in adipose content of muscle fibers with age, which would result in larger partition coefficients. Conversely, organs such as the lung and liver maintain a higher connective tissue mass (collagen) per unit volume with age (*42*). As a result, solubility would potentially decrease as a function of age. Results from our study support this hypothesis and reveal a consistent decrease in liver and lung solubility in the 1-day, 1-month, and 2-year exposure groups (Table 5).

Applications to Risk Assessment

Although significant effort has been put forth to characterize the disposition and metabolism associated with methylene chloride in young healthy animals (*3,43,44*), age-related changes in MFO and GST activity, as well as in physiological parameters, may be more important from a risk assessment perspective. According to Andersen et al. (*3*), the dose surrogate calculated from the amount metabolized by the GST pathway can be directly related to the carcinogenic risk of methylene chloride exposure; therefore, any physiologic or metabolic changes that result in an increase or decrease in the dose surrogate will directly impact the risk of methylene chloride-induced carcinogenesis. For example, the net effect of the increase in apparent V_{maxc} between the 1-day and 2-year groups, while holding K_{fc} constant, results in 18% decrease in the GST dose surrogate to both liver and lung. Furthermore, any decrease in the apparent K_{fc} with age would result in an additional and more dramatic decrease in the dose surrogate.

Age-related changes in disposition may also assist in interpreting the concurrent long-term carcinogenicity study for methylene chloride, which included various exposure regimens and stop exposures (*8*). Specifically, these studies revealed relatively high incidences of hepatocellular and pulmonary adenomas/carcinomas when methylene chloride was administered for durations of 26, 52, and 78 weeks prior to exposure to uncontaminated air for a total of 104 weeks (*8*). In contrast, relatively low incidences of hepatocellular and pulmonary ademonas/carcinomas were observed in mice when uncontaminated air was administered for the identical durations prior to methylene chloride (*8*). Although not conclusive, these changes in tumor incidence correlate well with a shift in methylene chloride metabolism toward the MFO pathway with increasing age and suggest that age at exposure may be an important risk factor in methylene chloride-induced carcinogenesis.

In conclusion, age and prior exposure to methylene chloride can produce notable changes in disposition and metabolism and may represent important factors in the interpretation of toxicologic and pharmacokinetic data. For methylene chloride specifically, further study is needed for age-related changes in GST metabolism for both interpretation of animal studies and identification of risk to humans.

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